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Differentiation of a human neural stem cell line on three dimensional cultures, analysis of microRNA and putative target genes --Manuscript Draft--

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Abstract:	Neural stem cells (NSCs) are capable of self-renewal and differentiation into neurons, astrocytes and oligodendrocytes under specific local microenvironments. In here we present a set of methods used for three dimensional (3D) differentiation and miRNA analysis of a clonal human neural stem cell (hNSC) line, currently in clinical trials for stroke disability (NCT01151124 and NCT02117635, Clinicaltrials.gov). HNSCs were derived from an ethical approved first trimester human fetal cortex and conditionally immortalized using retroviral integration of a single copy of the c-mycERTAM construct. We describe how to measure axon process outgrowth of hNSCs differentiated on 3D scaffolds and how to quantify associated changes in miRNA expression using PCR array. Furthermore we exemplify computational analysis with the aim of selecting miRNA putative targets. SOX5 and NR4A3 were identified as suitable miRNA putative target of selected significantly down-regulated miRNAs in differentiated hNSC. MiRNA target validation was performed on SOX5 and NR4A3 3'UTRs by dual reporter plasmid transfection and dual luciferase assay.
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Dear Eric Veien,

We are submitting a manuscript describing the protocols used for the characterization of human neural stem cell (hNSC) differentiation on a three dimensional (3D) culture system; the evaluation of changes in microRNA (miRNA) expression that occurs during differentiation using a stem cells and developmental pathways focused miRNA PCR array; computational analysis for miRNA target prediction and validation of target mRNAs by reporter plasmid transfection and dual luciferase assay.

The results obtained using the described laboratory techniques provide new insight into the identification of specific miRNAs implicated in hNSC differentiation. These strategies may be exploited to optimize *in vitro* hNSC differentiation potential for use in pre-clinical studies and future clinical applications.

All authors were employed by ReNeuron Ltd at the time of the study. Study design, reagents and procedures were all determined and/or supplied by ReNeuron Ltd.

Best Wishes,

Lara Stevanato, PhD

TITLE:

Differentiation of a human neural stem cell line on three dimensional cultures, analysis of microRNA and putative target genes.

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SHORT ABSTRACT:

With the intent of characterizing changes in miRNAs on differentiated human neural stem cells (hNSCs) we describe hNSC differentiation on a three dimensional system, the evaluation of changes in microRNA expression by miRNA PCR array, and computational analysis for miRNA target prediction and its validation by dual luciferase assay.

LONG ABSTRACT:

Neural stem cells (NSCs) are capable of self-renewal and differentiation into neurons, astrocytes and oligodendrocytes under specific local microenvironments. In here, we present a set of methods used for three dimensional (3D) differentiation and miRNA analysis of a clonal human neural stem cell (hNSC) line, currently in clinical trials for stroke disability (NCT01151124 and NCT02117635, Clinicaltrials.gov). HNSCs were derived from an ethical approved first trimester human fetal cortex and conditionally immortalized using retroviral integration of a single copy of the c-mycER^{TAM} construct. We describe how to measure axon process outgrowth

of hNSCs differentiated on 3D scaffolds and how to quantify associated changes in miRNA expression using PCR array. Furthermore we exemplify computational analysis with the aim of selecting miRNA putative targets. SOX5 and NR4A3 were identified as suitable miRNA putative target of selected significantly down-regulated miRNAs in differentiated hNSC. MiRNA target validation was performed on SOX5 and NR4A3 3'UTRs by dual reporter plasmid transfection and dual luciferase assay.

INTRODUCTION:

Stem cell research plays a central role in regenerative medicine, which also spans the disciplines of tissue engineering, developmental cell biology, cellular therapeutics, gene therapy, and biomaterials (scaffolds and matrices). Stem cells are important in both organ development and tissue repair; understanding how stem cells work is pivotal for developing new stem cell based treatments ¹. CTX0E03, is a clonal, conditionally immortalized human neural stem cell (hNSC) line ² isolated from first trimester human fetal cortex. CTX0E03 has defined quality characteristics that are essential for clinical cell banking under good manufacturing practice (GMP) ³. hNSCs can spontaneously differentiate into neurons, glia, and oligodendrocytes and have been proven to ameliorate neurological deficits when transplanted in a rodent model of focal ischemia ^{2,4,5}. The CTX0E03 hNSCs are currently in clinical trials for stroke disability (NCT01151124 ⁶ and NCT02117635, Clinicaltrials.gov).

MiRNAs have an average 22 nucleotides in length and are proven regulatory molecules ⁷, they do not encode proteins, but rather bind 3 prime untranslated region (3'UTR) of mRNAs, regulating their stability and translation into proteins. MiRNAs are reported to participate in the regulation of a number of cellular processes, including development, proliferation, and differentiation ⁸. Several miRNAs have been implicated in regulating the fate and specification of neural stem cells ⁹.

In two dimensional (2D) standard culture environment the complexity of the *in vivo* environment is not matched, and consequently the induction and regulation of hNSC differentiation is not optimal. *In vivo*, cells are surrounded by a three dimensional (3D) microenvironment composed by other cells and extracellular ligands, including many types of collagens, laminin, and other matrix proteins (extracellular matrix, ECM). Previous studies have reported that the architectural topography of the materials mimicking ECMs and their geometry influence cell phenotype and fate ¹⁰⁻¹⁵. A number of commercially available 3D scaffolds are available; we used a highly porous polystyrene scaffold engineered with a well-defined and uniform architecture into a 200 μ m thick membrane which provides a 3D space into which cells can invade and differentiate ¹⁶⁻¹⁸.

Herein 2D and 3D differentiation assays are used to assess axon process outgrowth. Furthermore we describe a method to profile the miRNA expression of a clinical grade hNSC line to further investigate miRNA effects on its differentiation. The methods in here illustrated are based on the ones originally described in ¹⁹.

PROTOCOL:

1. HNSC differentiation on three dimensional (3D) culture

NOTE: Isolate and derive hNSCs, from an ethical approved tissue, described in a previous publication². Please follow the ethical and institutional guidelines while following this procedure.

1.1) 3D culture using Alvetex® 12 well plate

1.1.1) Use aseptic technique throughout the procedure. In a biological safety cabinet, re-hydrate scaffolds by adding 2 ml/well of 70% ethanol prepared using water for irrigation (WFI). Avoid touching the scaffolds by dispensing the 70% ethanol down the wall of each well.

1.1.2) Carefully aspirate the 70% ethanol and immediately wash the scaffolds with 2 ml/well DMEM: F12 for one minute. Repeat the wash procedure twice. Carefully aspirate the DMEM: F12 after the final wash.

1.1.3) Coat scaffolds by adding 2 ml/well of laminin (10 µg/ml) in DMEM: F12. Incubate plate at 37 °C in a 5% CO₂ humidified incubator for a minimum of 2 hours. Wash plate with warm DMEM: F12.

1.1.4) Seed each scaffold with approximately 500,000 hNSCs in a volume of 150 µl of RMM+GFs.

1.1.5) Incubate the plate for 3 hrs at 37 °C in a 5% CO₂ humidified incubator to allow cells to settle into the scaffolds.

1.1.6) Add 3.5 ml of RMM to each well taking care not to dislodge cells from scaffolds.

1.1.7) Incubate the plate for 7 days; replace RMM medium (3.5 ml/well) after 1 day (first feeding) and again after 2 -3 days from the first feeding.

1.1.8) After 7 days, remove medium and fix cells with 4% paraformaldehyde (PFA) for immunocytochemistry (ICC) or add 500 µl/well of lysis reagent for total RNA extraction (store plate at ≤-80 °C or proceed to total RNA extraction).

1.2) Measurement of axon process outgrowth

1.2.1) Stain 4% PFA fixed differentiated hNSCs according to a standard ICC protocol²⁰ using β3-tubulin (TUBB3) primary antibody detected with a fluorochrome conjugated secondary antibody. Counterstain cell nuclei with Hoechst (1 µM).

1.2.2) Capture representative images of β3-tubulin stained cells using a fluorescent microscope; save images as tiff or jpeg. Measure of axon process outgrowth using image-Pro Plus 7 software (Media Cybernetics) measurement tool.

1.2.3) Open the image, select measurements option in the tool bar, and select trace feature.

1.2.4) To start the trace, select the start point on the axon outgrowth by clicking the mouse button. Trace along the entire length of the axon outgrowth; right click to finish each trace. Repeat to measure all axon outgrowths within the field of view.

1.2.5) Express measurements as pixels or μm (pending calibration set up). Export length measurements to a spreadsheet program for sample comparative and statistical analysis.

2. MiRNA expression using a stem cells and developmental pathways focused miRNA PCR array

lysis reagent

2.1) miRNA total RNA extraction

2.1.1) Perform miRNA extraction on scaffolds and 2D grown samples (differentiated and undifferentiated, hNSC control).

2.1.2) Thaw scaffolds, inserted in a 12 well plate, if required. Combine lysis reagent contents (500 μl) of 2 wells in the same 1.5 ml Eppendorf tube, final volume 1 ml of Lysis reagent. Take care to leave the scaffold in the well.

2.1.3) For previously collected as dry cell pelleted 2D grown samples (differentiated hNSC 2D cultures and undifferentiated control) add 1ml of lysis reagent and transfer contents into 1.5 ml microcentrifuge tube.

2.1.4) Homogenize each test sample using a 1 ml syringe and a short 20 to 25 gauge needle. Pass the lysate through the needle using a 1ml syringe up to 5 times until a homogeneous lysate is achieved.

2.1.5) Add 200 μl of chloroform to each Eppendorf tube and cap securely. Invert and vortex tubes to mix contents.

2.1.6) Centrifuge each 1.5 ml microcentrifuge tube containing homogenate for 15 min at $\geq 12,000 \times g$.

2.1.7) Transfer the upper aqueous phase of each sample (avoid transferring any interphase, pink coloured liquid) to a fresh 1.5 ml microcentrifuge tube containing 750 μl of 100% ethanol. Mix thoroughly by inversion.

2.1.8) Pipette up to 700 μl of each test sample, including any precipitate, into a mini column in a 2 ml collection tube. Close the lid and centrifuge at $\geq 8000 \times g$ for approximately 30 sec at room temperature. Discard the flow-through. Repeat this step using the remainder of the sample.

2.1.9) Perform a DNA digest for 15 min.

2.1.10) Add 700 µl buffer RWT to the mini column and centrifuge for approximately 30 sec at $\geq 8000 \times g$. Discard the flow-through.

2.1.11) Wash mini column by adding 500 µl buffer RPE and centrifuging for approximately 30 sec at $\geq 8000 \times g$. Discard the flow-through. Repeat wash. Centrifuge at $\geq 12,000 \times g$ for 1 min to dry the membrane further.

2.1.12) Elute total RNA in a 1.5 ml Eppendorf tube by adding 40 µl of RNase-free water to the mini column and centrifuging for 1 min at $\geq 8000 \times g$. Measure total RNA concentration with the aid of an appropriate spectrophotometer.

2.2) miRNA cDNA preparation

2.2.1) Prepare the reverse-transcription master mix. Each sample requires 4 µl of 5x miScript HiSpec buffer, 2 µl of 10x nucleic mix, 2 µl miScript reverse transcriptase mix.

2.2.2) Aliquot 8 µl of master mix in a PCR tube, add required volume of total RNA (250 ng), and add RNase-free water to a final volume of 20 µl.

2.2.3) Incubate for 60 min at 37 °C. Incubate for 5 min at 95°C to inactivate miScript Reverse Transcriptase Mix.

2.2.4) Add 200 µl RNase-free water to each 20 µl reverse-transcription reaction, store at –20 °C, or proceed with real-time PCR immediately.

2.3) Stem cells and developmental pathways focused miRNA PCR array

2.3.1) Prepare the PCR components mix in a 15 ml tube by adding 1375 µl of 2x SYBR green master mix, 100 µl miRNA cDNA preparation, 275 µl of 10x miScript Universal Primer, and 1000 µl of RNase-free water (total volume 2750 µl).

2.3.2) Transfer PCR components mix on a PCR loading reservoir. Carefully remove the 96 well plate PCR array from its sealed bag.

2.3.3) Add 25 µl PCR components mix to each well of the PCR Array using an 8-channel pipettor, or a 12-channel pipettor using only 8 tips. Change pipet tips following each pipetting step to avoid cross-contamination between the wells.

2.3.4) Seal plate with optical adhesive film, and centrifuge for 1 min at 1000 g at room temperature (15–25°C) to remove bubbles. Visually inspect the plate from underneath to ensure no bubbles are present in the wells.

2.3.5) Place the 96 well plate PCR array in the real-time cycler.

2.3.6) Program the real-time cycler accordingly: 1 cycle at 95 °C for 10 min, and 45 cycles for 15 s at 95 °C, and 1 min at 60 °C set a single (fluorescence data collection).

2.3.7) Export the Ct values for all wells to a blank Excel spreadsheet for use with PCR Array Data Analysis Template Excel and web-based software. NOTE: Software is available at www.SABiosciences.com/pcrarraydataanalysis.php.

3. Computational analysis for miRNA target prediction and validation of target mRNAs by reporter plasmid transfection and dual luciferase assay.

3.1) Computational analysis for miRNA target prediction

3.1.1) Browse PicTar (<http://pictar.mdc-berlin.de/>)²¹, online available algorithm for the identification of miRNA target prediction mRNAs.

3.1.2) Click on click here for PicTar prediction on vertebrates, a new page will open.

3.1.3) In the new page, PicTar web interface, choose species in the drop down menu and insert miRNA of interest in the miRNA ID box. Click search for targets of a miRNA.

3.1.4) Observe a list of target mRNAs ranked by a PicTar score.

NOTE: PicTar computes a score reflecting the likelihood that a given UTR will be targeted by the selected miRNA, and based on seed complementarity, thermodynamics and a combinatorial prediction for common targets in sets of co-expressed miRNAs²².

3.1.5) Similarly retrieve list of target mRNAs using DIANA-LAB (<http://diana.cslab.ece.ntua.gr/microT/>)²³, and TargetScan (<http://www.targetscan.org/>)²⁴, alternative on line available algorithms.

3.1.6) Compile a comparative table of miRNAs based on ranking obtained using different software tools. PicTar ranks by score, DianaLab by precision (based on signal to noise ratio and a precision score for the evaluation of the significance of the predicted results), and TargetScan by aggregate PCT (based on seed complementarity, thermodynamic free energy of binding, conservation over different species), see table 1.

3.2 Validation of target mRNAs by reporter plasmid transfection and dual luciferase assay.

NOTE: 3' UTR luciferase reporters for the validation of target mRNA has been previously described²⁵.

3.2.1 Transfection

3.2.1.1) Seed HeLa cells at a density of 10⁵ cells/well in 24-well plate.

3.2.1.2) After 24 h co-transfect HeLa cells with 1.5 µl of transfection reagent and either 100 ng

of NR4A3 3' UTR or SOX5 3' UTR plasmid, as well as 20 nM miRNA mimics (hsa-miR-96-5p for SOX5 3' UTR, and hsa-miR-7-5p, and hsa-miR-17-5p for NR4A3 3' UTR respectively) per well.

3.2.1.3) Co-transfect control wells with NR4A3 3' UTR or SOX5 3' UTR plasmid and fluorochrome labelled negative control siRNAs. The transfection of control wells with fluorochrome labelled negative control siRNAs allows evaluation of transfection efficiency and accounts for mimic miRNA 3'UTR bind specificity.

3.2.2 Measurement of dual luciferase activities

3.2.2.1) Measure Firefly and Renilla luciferase activities 24 hr post transfection. Remove cell growth medium.

3.2.2.2) Prepare working solution I by adding 50 µl of substrate I into 10 ml of Solution I; mix thoroughly. Add 300 µl of working solution I into each 24 well.

3.2.2.3) Transfer the contents of each 24 well into 3 wells of a 96 white plate, 100 µl each. Wait 10 min and measure Firefly luciferase in luminometer set for luminescence acquisition.

3.2.2.4) Prepare working solution II by adding 50 µl of substrate II into 10 ml of solution II; mix thoroughly. Add 100 µl of working solution I into each 96 well already containing 100 µl of working solution II.

3.2.2.5) Wait 10 min and measure Renilla luciferase in luminometer set for luminescence acquisition. Calculate the ratio of luminescence from the Firefly luciferase to the Renilla luciferase.

REPRESENTATIVE RESULTS:

In Figure 1 is visualized the investigation and quantification of hNSC differentiation on 3D scaffolds compared with traditional flat surface cultures (2D) and expressed as axon process outgrowth measurements. In Figure 2 is represented the workflow and the results for the miRNA quantification using stem cells and developmental pathways focused miRNA PCR array to investigate differential expression of miRNAs in 2D and 3D differentiated hNSCs compared with undifferentiated control. Following miRNA comparative analysis between differentiated and undifferentiated hNSCs, and computational analysis for target prediction, SOX5 and NR4A3 (NOR1) were identified as putative target mRNAs. To study the direct interaction between the miRNAs and their putative site on the 3' UTR mRNA we used 2 commercially available plasmids containing either SOX5 or NR4A3 3' UTR sequence inserted downstream of the firefly luciferase reporter gene, and containing in the same plasmid renilla luciferase gene for normalization. The representative results of 3' UTR luciferase activity down-regulation is presented in Figure 3.

Figure 1: Differentiation of hNSCs on 3D scaffolds, and measurement of axon processes outgrowth.

A) Following standard ICC staining for β 3-tubulin (green) and nuclei counterstained with Hoechst (blue) representative microphotographs were acquired and measurement of axon

processes were performed with the aid of an image analysis software. **B)** Diagrams reporting the measurement of axon process outgrowth performed in 2D and 3D differentiated hNSCs for 1 (1W) or 3 weeks (3W).

Figure 2: Schematic workflow of miRNA profiling in differentiated hNSCs.

Total RNA, including miRNAs, was extracted from either differentiated hNSCs on 3D scaffolds and standard 2D cultures, or undifferentiated control. 250 ng of total RNA were retro-transcribed with a method that facilitates the selective conversion of mature miRNAs into cDNA suitable for miRNA quantification with human cell differentiation and development miScript miRNA PCR array. The geo-mean of SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, and RNU6-2 was used for data analysis based on the $2^{-\Delta\Delta Ct}$ method. Significant changes were defined as +/- 1.5 fold up and down regulation to a statistically significant extent. Data were analyzed using web-based software available at www.SABiosciences.com/pcrarraydataanalysis.php. Results are expressed as a clustergram. Modified from figure 3¹⁹.

Figure 3: Validation of miRNA target predicted mRNA using a dual luciferase report assay.

A) Representative diagram of a dual luciferase reporter plasmid used as a tool to validate 3'UTR sequence as a direct target of a miRNA. **B)** Representative micrograph showing transfection efficiency. **C)** Signals, expressed as relative luciferase activity, from human 3'UTR reporters of SOX5 and NR4A3 genes were significantly knocked down in the presence of hsa-miR-96, and hsa-miR-7 and 17 miRNA mimics respectively. Modified from figure 5¹⁹.

Table 1: List of miRNA target prediction mRNAs.

Representative table of miRNAs, target predictive genes, and algorithms analysis (prediction/score/aggregate provided using Diana Lab, PicTar, and TargetScan respectively). Modified from table 3¹⁹.

DISCUSSION:

The development of new *in vitro* assays to assess and improve the differentiation of stem cells has always presented a challenge for the investigation of their functionalities and therapeutic capabilities. Long term and stable attachment of hNSCs, required for the development of a robust 3D *in vitro* differentiation assay, was addressed by testing several 3D substrates with different characteristics in term of materials and structures. As part of the assay development we also assessed optimal cell seeding concentration and identified the requirement for the scaffolds to be laminin coated. Although our hNSCs can spontaneously differentiate upon 4-OHT and growth factor removal, the implementation of a 3D culture system showed a significant improvement in their differentiation capability as measured by axon process outgrowth when compared to standard 2D differentiated hNSCs.

To investigate the effect of 3D induced differentiation on hNSC miRNA expression we used a PCR array. Compared with standard chip microarray PCR array offers the advantages of a direct quantification and validation of miRNA of interest. Although the PCR array is limited in term of total number of miRNA per array, for example in here less than 96, it can be tailored to specifically include miRNAs of interest, in our case previously reported in stem cell

development. The expression of pathway focused miRNAs was profiled in 3D and 2D differentiated hNSCs compared with undifferentiated hNSCs. The most significantly down-regulated miRNAs were selected and analyzed to assess their putative target mRNAs with the intent of determining their functionality and biological interpretation using online available algorithms (DIANA Lab, PicTar, and TargetScans).

A single miRNA can recognize hundreds of targets and for this reason we validated miRNA interactions with 3' UTR mRNAs that may be suitable candidates in axonal regulation. NR4A3, a target of the hsa-miR-7, and miR-17 family, is a member of the NR4A family nuclear receptors which, depending on their level of expression, are involved in the differentiation, survival, apoptosis and regulation of hippocampal axon guidance²⁶. Similarly SOX5, a target of hsa-miR-96, is reported to control cell cycle progression in neural progenitors²⁷ axon length²⁸, migration, post-migratory differentiation, and projections of neurons²⁹. Both SOX5 and NR4A3 were identified as a direct target mRNA of hsa-miR-96, and hsa-miR-7 and 17 respectively by dual luciferase reporter assays. Dual luciferase (Firefly and Renilla) relies on two different reporter genes in the same plasmid and offers an improved system allowing normalization for effects caused by suboptimal transfection and cytotoxic effects compared with a single luciferase plasmid system. As part of our assay development we also optimized our transfection conditions in order to obtain high efficiency.

The protocols described herein may be exploited to further optimize and characterize *in vitro* hNSC differentiation which may be implemented in transplantation protocols for pre-clinical studies and future clinical applications.

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DISCLOSURES:

All authors are employees, stock and/or stock option holders in ReNeuron Ltd or its parent company.

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Figure 1
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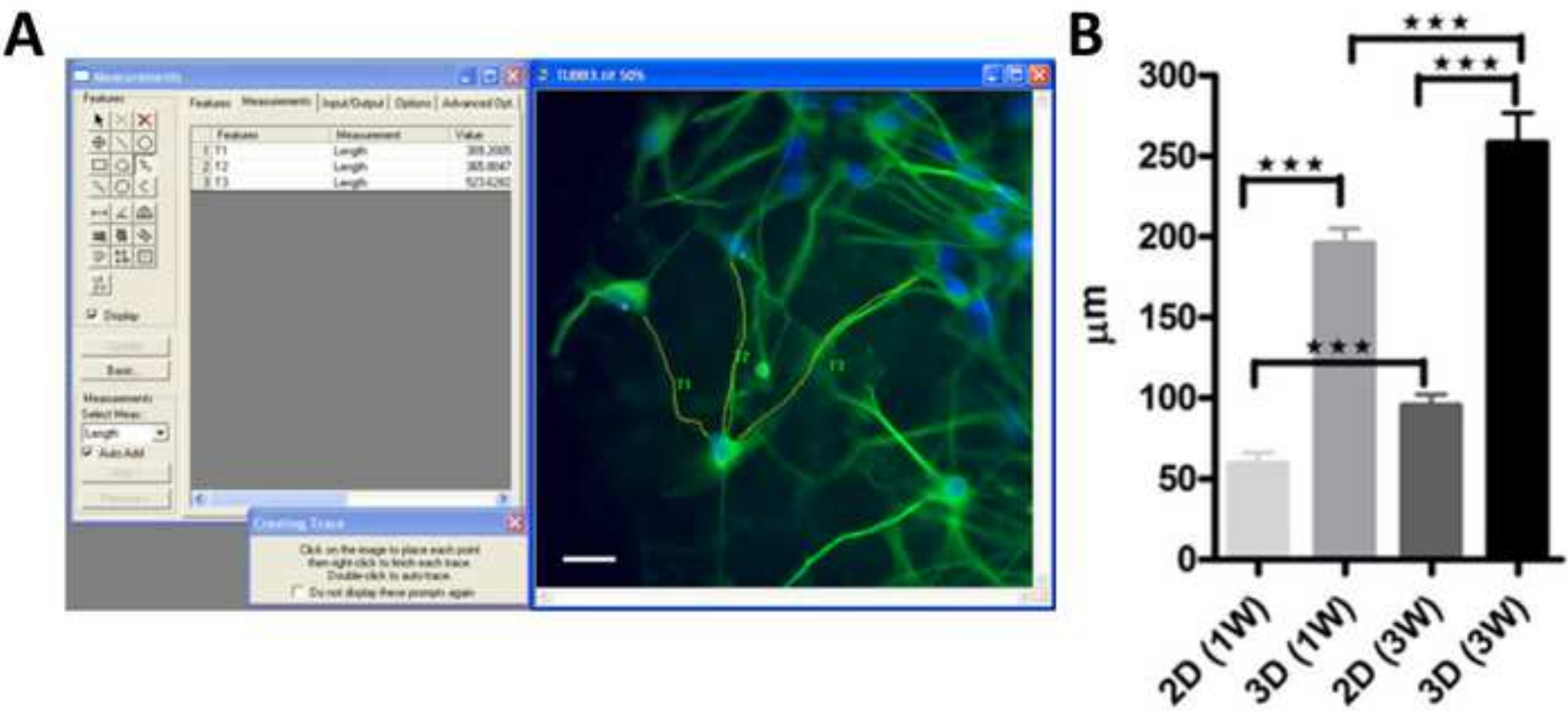


Figure 2
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Pathways focused miRNA PCR array workflow

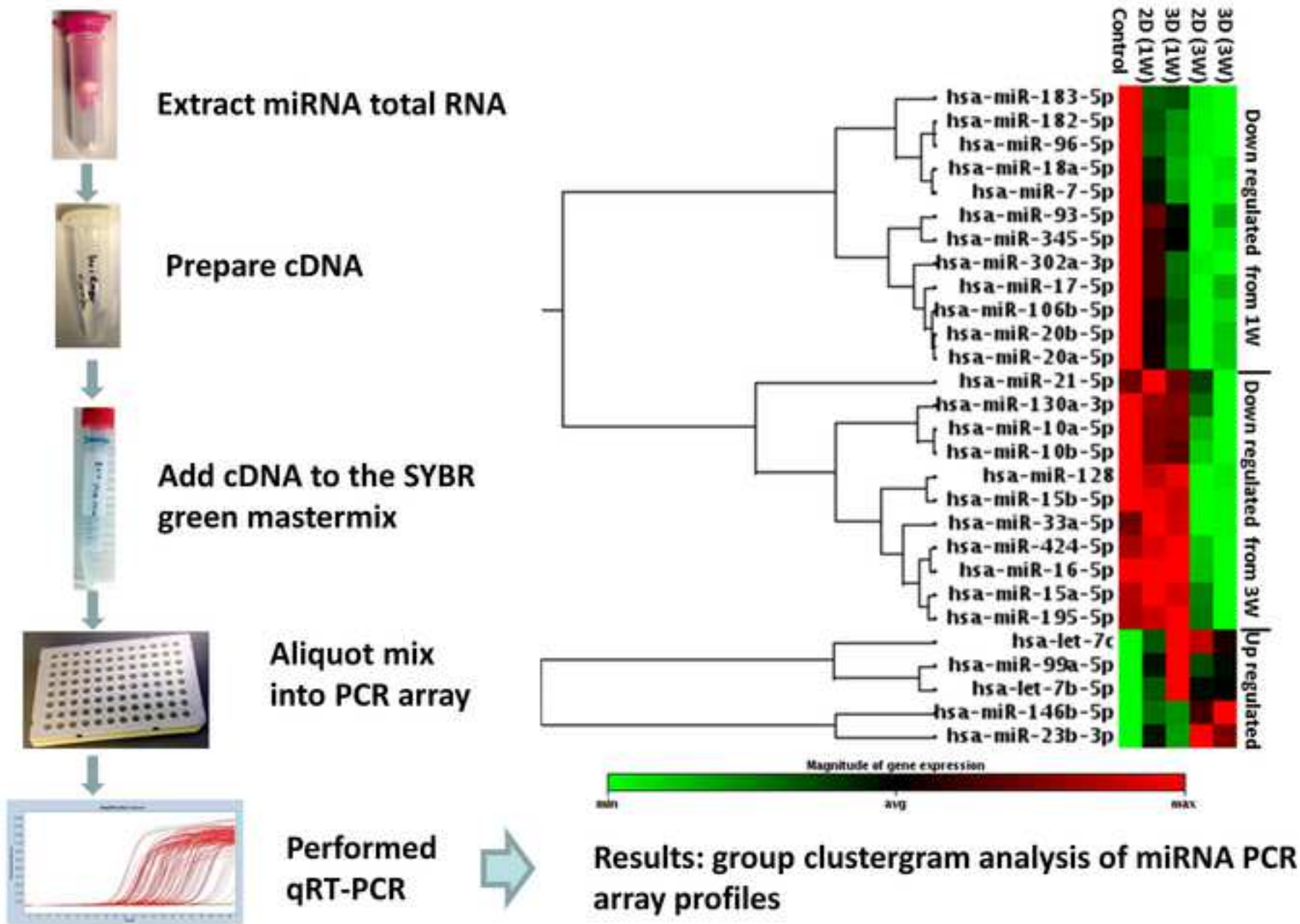


Figure 3
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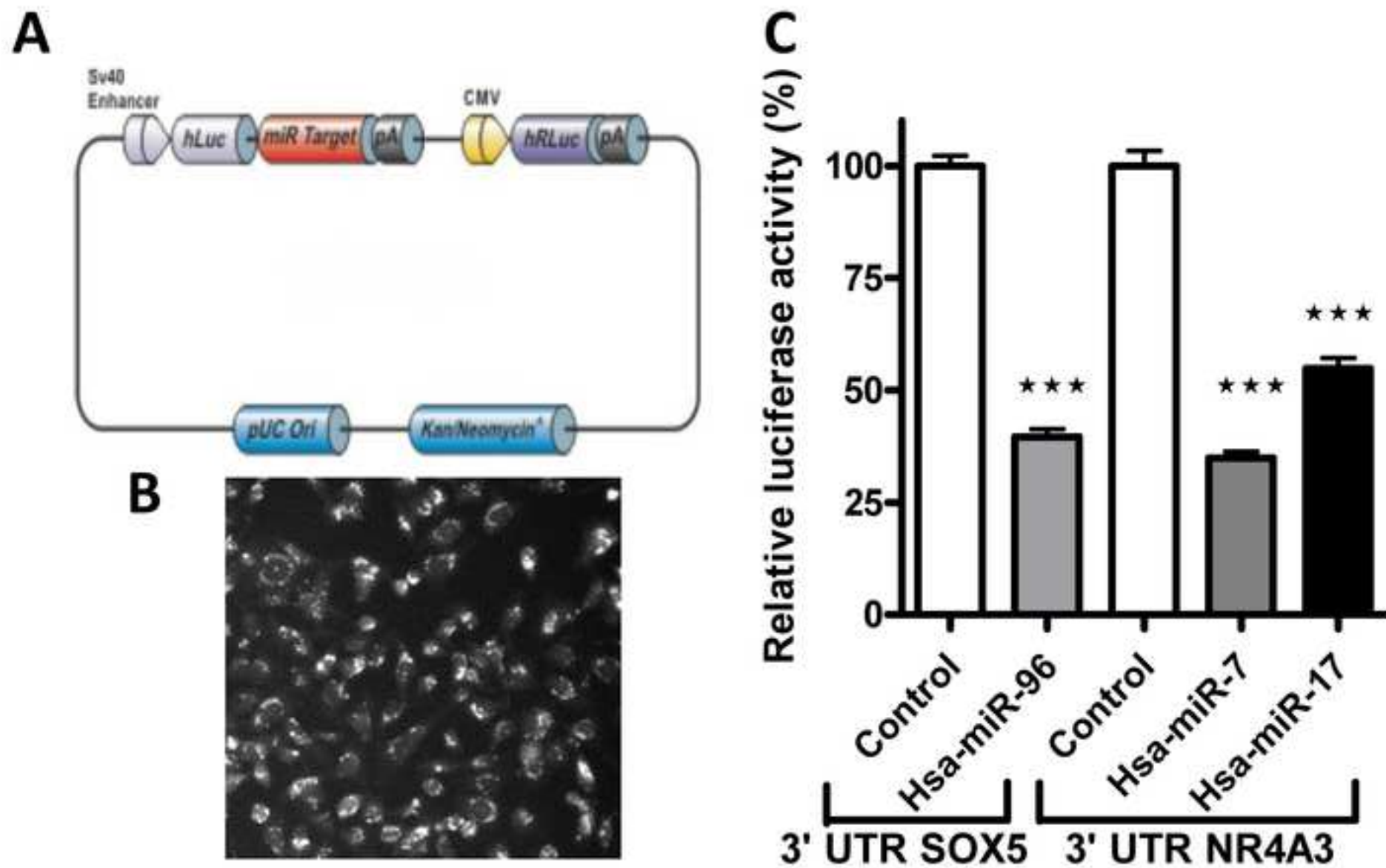


Table 1
[Click here to download Table: table 1 Stevanato L.xlsx](#)

		DIANALAB		PicTar	TargetScan
miRNA	Target gene	Target sites/genes found	Precision	Score	Aggregate P _{CT}
hsa-miR-96	SOX5	1214/763	1	6.74	0.94
hsa-miR-183	DUSP10	302/190	0.72	4.75	0.85
hsa-miR-302a	NR4A3	863/473	0.84	3.05	NF
hsa-miR-182	FOXN3	1358/794	0.94	NF	0.96
hsa-miR-7	NR4A3	NF	NF	1.61	0.17
hsa-miR-7	FOXN3	399/136	0.17	NF	0.88
hsa-miR-20a	NR4A3	1650/841	0.85	5.87	0.63
hsa-miR-20b	NR4A3	1955/973	0.9	5.87	0.63
hsa-miR-17	NR4A3	1928/961	0.9	5.38	0.63+0.37
hsa-miR-20a	EIF4G3	1650/841	0.97	NF	NF
hsa-miR-20b	EIF4G3	1955/973	0.94	NF	NF
hsa-miR-17	EIF4G3	1928/961	0.94	NF	NF

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
DMEM:F12	Invitrogen	21331-020	For RMM medium preparation
Human Albumin Solution	Baxter healt	1501052	For RMM medium used at a final concentration of 0.03%
Transferrin, Human recombinant	Sigma	T8158	For RMM medium used at a final concentration of 5 µg/ml
Putrescine DiHCl	Sigma	P5780	For RMM medium used at a final concentration of 16.2 µg/ml
Insulin, Human recombinant	Sigma	I9278	For RMM medium used at a final concentration of 5 µg/ml
Progesterone	Sigma	P6149	For RMM medium used at a final concentration of 60 ng/ml
L-Glutamine	Invitrogen	25030-24	For RMM medium used at a final concentration of 2 mM
Sodium Selenite	Sigma	S9133	For RMM medium used at a final concentration of 40 ng/ml
bFGF	Peprotech	AF-100-15	To be added to RMM medium, used at a final concentration of 10 ng/ml
EGF	Peprotech	AF-100-188	To be added to RMM medium, used at a final concentration of 20 ng/ml
4-OHT	Sigma	H7904	To be added to RMM medium, used at a final concentration of 100 nM
Laminin	AMS Biotect	3400-010-10	
TrypZean/EDTA	Lonza	BE02-034E	
Water for irrigation	Baxter Health	UKF7114	
Defined Trypsin Inhibitor (DTI) (store at -20°C)	Invitrogen	R007-100	
Alvetex 12 well plate	Reinervate	AVP002	
Ethanol	Merck	1.00986.1000	
βIII tubulin antibody	Sigma	T8660	
Hoechst	Sigma	B2261	
miRNeasy mini kit	Qiagen	217004	
RNase free DNase	Qiagen	79254	
Chloroform	Sigma	C7559-5VL	
miScript II RT Kit	Qiagen	218160	
SYBR green PCR kit	Qiagen	218073	
Cell Development & Differentiation medium	Qiagen/ SAE	MIHS-103Z	
Lightcycler 480 white 96 plate	Roche	4729692001	
Lightcycler 480 sealing foil	Roche	4729757001	
Lipofectamine 2000	Invitrogen	11668-027	
Vector NR4A3 miRNA 3' UTR target clone	GeneCopeia	HmiT019538-MT0	
Vector SOX5 miRNA 3' UTR target clone	GeneCopeia	HmiT017632-MT01	
Allstars negative control siRNA AF 48496	Qiagen	1027284	MiRNA transfection control
Luc-Pair miR Luciferase Assay	GeneCopeia	LPFR-M010	
Lightcycler 480	Roche		

GloMax 96 microplate
luminometer

Promega



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Manuscript #:

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Author(s): Lara Stevanato, Caroline Hicks, and John D Sinden

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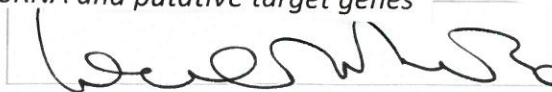
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Manuscript JoVE52410 'Differentiation of a human neural stem cell line on three dimensional cultures, analysis of microRNA and putative target genes' Authors' responses to editorial comments

Dear Eric Veien, Ph.D and Jaydev Upponi, Ph.D

Thank you for the evaluation of our manuscript entitled “Differentiation of a human neural stem cell line on three dimensional cultures, analysis of microRNA and putative target genes” for publication in Journal of Visualized Experiments

We found the editorial comments both insightful and helpful. Based on these comments we have made all of the appropriate changes/edits to our manuscript. A detailed response to each question follows below. Modifications to text are shown in track changes in the Related Manuscript File.

Thank you for the opportunity to reconsider our manuscript for publication in Journal of Visualized Experiments

We look forward to hearing your response.

Warm regards,

Lara Stevanato, PhD

Your manuscript JoVE52410 'Differentiation of a human neural stem cell line on three dimensional cultures, analysis of microRNA and putative target genes' has been editorially reviewed and the following comments need to be addressed.

Editorial comments:

1.Your current short abstract is above our 50 word limit; please make sure the short abstract is between 10 to 50 words.

Modified as request, current word count is 49.

2.Please state the goal of your protocol in the Short abstract.

A goal was stated in the Short abstract: ***With the intent of characterizing changes in miRNAs on differentiated human neural stem cells (hNSCs).***

3.Your current long abstract is under our 150 - 300 word limit; please modify your long abstract in both the manuscript document and your editorial manager account.

Modified as request, current word count is 158.

4.Please add a one line space between each step and sub-steps of your protocol section.

One line space has been added between each step and sub-steps of your protocol section.

5. Please adjust the numbering of your protocol section to follow JoVE instructions for authors, 1. should be followed by 1.1) and then 1.1.1) if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one line space between each protocol step.

Modified as request, please directly refer to manuscript document.

6. Please include the paragraph mentioned below step 1 in the step wise protocol section. Please be sure the write the protocol in imperative tense. For instance, "Isolate HNSCs from ..." etc. Please perform similar action in section 2.

The short protocol introduction was removed and replaced by a shorter statement:

The isolation and derivation of hNSCs, from an ethical approved tissue, are described in a previous publication² .

7. Please include a ethics statement below the protocol section and before the beginning of the numbered protocol steps.

The ethical statement has been included: The isolation and derivation of hNSCs, from an ethical approved tissue

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All the commercial sounded language has been removed from the manuscript, and figure.

9. Please write step 3.1 in imperative tense.

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10. Please use numbering and correct format and write the paragraph below step 3.2 stepwise in imperative tense.

Modified as request, please directly refer to manuscript document.

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non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

The most important portions of the protocol for the video inclusion were highlighted in yellow.

12. Please make sure that the “Discussion” section covers the following points running between 3 – 6 paragraphs.

- a. Critical steps within the protocol.
- b. Modifications and troubleshooting.
- c. Limitations of the technique.
- d. Significance of the technique with respect to existing/alternative methods.
- e. Future applications or directions after mastering this technique.

We rephrased, expanded, and modified the discussion to address the above listed requirements.

Please refer directly to the manuscript document.

13. Please remove the embedded Materials Table from the manuscript. All tables should be uploaded to the Editorial Manager site in the form of Excel files. A description of the table should be included with the Figure legends. Please remove the TM/R marketing symbols.

The TM/R marketing symbols were removed from the document and table.

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The latest version of Jove style for EndNote was uploaded in the manuscript linked EndNote library. In regard to the DOI issue Jaydev Upponi, science editor, courteously offer to upload the DOI number in the manuscript.

Differentiation of a human neural stem cell line on three dimensional cultures, analysis of microRNA and putative target genes

To Jove Science Editor - Bioengineering

Dear Eric Veien, Ph.D.

Thank you for the evaluation of our manuscript JoVE52410R1.

Based on these comments we have made all of the appropriate changes/edits to our manuscript. A detailed response to each question follows below. Modifications to text are shown in track changes in the Related Manuscript File.

Thank you for the opportunity to reconsider our manuscript for publication in *Jove*

We look forward to hearing your decision.

Warm regards,

Lara Stevanato, PhD & Caroline Hicks, MSc

Reviewer number: 1

Reviewer #1:

Major Concerns:

Page 3: description of the measurement of axon outgrowth is vague. The Authors don't give information about the software used for the analysis and how to perform the analysis.

We have included the information about software used for the analysis and how to perform the analysis.

Page 6: target prediction using only one algorithm (PicTar) doesn't give enough information to identify miRNA target. Each algorithm gives different target considering different parameters e.g. binding energy and position of the seed sequence. The prediction will be more accurate if results from different software are corroborated. Authors need to explain this on the protocol.

We have included an explanatory statement for each listed algorithm, however for detailed information regarding specific algorithmic parameters we propose consultation of webpages and relevant referenced publications.

Page 6: to validate the binding of miRNA to the target mRNA using luciferase assay, the Authors need to also mutate the miRNA binding sites and repeat luciferase assay. This will confirm the specific binding of miRNA to the target 3'UTR

We have provided a clarification in the transfection experimental conditions. The transfection of control wells with fluorochrome labelled negative control siRNA allows evaluation of transfection efficiency and accounts for mimic miRNA 3'UTR binding specificity.

Minor Concerns:

In the discussion, the Authors should discuss troubleshooting of the protocols and eventually alternative to the described techniques.

We have modified the discussion to better underline troubleshooting and alternative techniques.

Reviewer #2:

Major Concerns:

This is a methods paper that essentially cobbles together a few kits, previously designed basic microscopy software, and other people's websites. They do describe a protocol using laminin to coat a commercially available 3D scaffold, in which case they claim to have done some optimization to increase the "reproducibility of hNSC attachment on the scaffolds", but that's not particularly groundbreaking. The method of deriving the cells is previously described. They then describe how to use free image analysis software made by someone else to measure axon outgrowth in images of cells (you trace them). The miRNA expression analysis uses a microRNeasy kit to isolate RNA followed by a miScript RT kit followed by a SYBR green qPCR kit. They may have made minor modifications but overall the protocols are those that come in the kit and in some cases they just copied and pasted them directly into this document. Next they explain how to use PicTar - a website used to predict microRNA targets. They then use commercially available vectors and a

luciferase kit to show microRNA regulation of putative targets - probably the most common way to do this - and detail the instructions that come with this kit.

In our opinion the major concerns raised are without constructive critique. We have never claimed that the techniques described in the paper are novel; we submitted the paper following a direct invitation from the Editor, Eric Veien, PhD. The manuscript describes a set of techniques that can be used to optimize NSC differentiation on 3D substrates compared with standard 2D cultures and provides a correlation with changes in miRNA expression and identification of putative mRNA targets.

We tested several scaffolds; the one presented in the manuscript produces the most robust cell differentiation and long term attachment. Additionally, in the field of molecular biology and specifically miRNAs analysis, all the investigations are based on commercially available tools (e.g. PCR array or chip microarray or any reagents for qRT-PCR, and report luciferase system) and any ad hoc optimization is primarily based on comparison of commercially available systems.

Minor Concerns:

The paper has lots of typos as well as awkward phrasing.

Corrections have been made to the text.